Reference: *Biol. Bull.* 175: 284–288. (October, 1988)

Fiber Types in the Limb Bender Muscle of a Crab (*Pachygrapsus crassipes*)

MICHAEL P. McDERMOTT AND PHILIP J. STEPHENS*

Villanova University, Department of Biology, Villanova, Pennsylvania 19085

Abstract. The bender muscle in the walking limb of the Pacific shore crab (*Pachygrapsus crassipes*) is composed of fibers with different structural (sarcomere length) and histochemical (NADH diaphorase and myofibrillar ATPase) properties. Slow fibers are located along the dorsal margin of the muscle and along the ventral margin in the distal portion of the muscle. The remaining bender muscle is composed of intermediate-type fibers, which can be differentiated into two groups based upon the pH sensitivity of the myofibrillar ATPase activity and the polysaccharide content of the fibers.

Introduction

Vertebrate motor neurons are considered to have a trophic influence on their target muscle fibers, since their physiological characteristics have a profound effect on the physiological properties and structural integrity of the muscle (Guth, 1968; Gutmann, 1976). Altering the firing activity or changing the motor supply can alter the contraction speed (Buller *et al.*, 1960; Buller and Lewis, 1965; Lomo *et al.*, 1974; Luff, 1975), calcium uptake (Sreter *et al.*, 1975), and energy metabolism (Buller *et al.*, 1969; Barany and Close, 1971; Pette, 1984) of the muscle fibers. Recently, work on invertebrates involving the fast excitor to the crayfish claw closer muscle has revealed that changing the firing patterns causes changes in the physiological (Lnenicka and Atwood, 1985; Pahapill *et al.*, 1986) and morphological (Lnenicka *et al.*, 1986) properties of the synapses (review: Atwood and Wojtowicz, 1986). As a prelude to a study of the effects of changing motor axon firing patterns on the properties of target muscle fibers, we have examined certain morphological and histochemical properties of the bender muscle in pristine walking limbs of the crab *Pachygrapsus crassipes*.

The skeletal muscles of crustaceans may be innervated by as few as three motor neurons (Wiersma and Ripley, 1952) and some evidence for trophic interactions between nerve and muscle comes from a close matching between motor neuron and muscle properties (Atwood, 1973). In the claw closer muscle, for example, there is a matching between muscle fiber type and the innervation patterns of the two excitatory motor neurons. In lobster, the innervation patterns of the two excitatory motor axons are matched with the oxidative capacity of the muscle fibers (Lang *et al.*, 1980). The oxidative capacity of fibers is low when innervated only by the fast axon, high when innervated only by the slow axon, and intermediate when innervated by both axons. In the crab closer muscle, a more detailed study has been performed on a small number of identifiable closer muscle fibers (Maier *et al.*, 1984). In this study the fast fibers in the crab closer muscle were divided into three sub-groups on the basis of the pH sensitivity of myofibrillar adenosine triphosphatase (ATPase) activity (Tse *et al.*, 1983). The slow and some fast (group II) closer muscle fibers are innervated by the fast and the slow excitatory axons, while the remaining fast muscle fibers are innervated by only the fast closer excitor.

In the present study we have examined certain structural and histochemical properties of fibers in the bender muscle in the walking limbs of the Pacific shore crab (*Pachygrapsus crassipes*). The limb bender muscle is innervated by two excitatory motor axons and by branches of the common inhibitor (Wiersma and Ripley, 1952). We show that the bender muscle is composed of slow and two types of intermediate fibers, and that these different types of fibers are regionally distributed within the bender muscle.

Materials and Methods

Crabs (*P. crassipes*) were obtained from the Pacific Biomarine Laboratories, Venice, California, and were

Received 13 October 1987; accepted 27 July 1988.

* To whom all correspondence should be sent.

kept in the laboratory at 24°C. The animals were kept individually, and feeding (Purina rabbit chow) and subsequent seawater changes were performed every 2 to 3 days. Observations were made using autotomized, second and third walking limbs removed from crabs that had been acclimated to laboratory conditions for at least 4 weeks.

Certain histochemical properties were examined in frozen sections of the bender muscle. The cuticle of the carpus was reduced in thickness with a dental drill. The stretcher muscle was removed, and the bender muscle plus the remaining thin cuticle were mounted on a chuck in *Histo Prep* (Fisher Scientific) and immersed in liquid nitrogen; the tissue was allowed to equilibrate to −25°C in the cryostat. Sections (20 μm thick) were mounted on glass slides and air dried for 15 to 60 min.

Sections were stained for activity of the mitochondrial enzyme nicotinamide dehydrogenase (NADH) diaphorase using the method of Ogonowski and Lang (1979), and for calcium-activated myofibrillar adenosine triphosphatase (ATPase) activity with acidic or basic preincubation, using a modified procedure of Padykula and Herman (1955). Acidic preincubation was performed in a solution of 100 m*M* KCl and 100 m*M* Na-acetate at pH 5.0 for 10 min at room temperature. Preliminary experiments using pH 4.6 pre-incubation (Maier *et al.*, 1984) did not produce differentiation of muscle fibers in sections of the bender muscle. Alkaline preincubation was carried out in a solution of 18 m*M* $CaCl_2$ and 25 m*M* Na barbitol at pH 9.4 for 5 min, then transferred to the same solution containing 0.05% mercaptoethanol for 20 s. For both preincubation regimes, myofibrillar ATPase activity was determined as described by Maier *et al.* (1984). Sections were dehydrated in a graded series of ethanol, cleared in xylene, and mounted in Permount.

The polysaccharide content of bender muscle fibers was determined in unfixed, frozen sections using the method of Lillie and Fullman (1976); no counterstain was used.

Sarcomere length measurements were made from single bender muscle fibers fixed at resting length (O'Connor *et al.*, 1982). The cuticle over the stretcher muscle was removed. The underlying stretcher muscle, limb nerve, and connective tissue layer were carefully removed to expose the bender muscle. The preparation was bathed in a calcium-free, high magnesium crab saline for 1 h in an attempt to reduce muscle contraction during fixation. The carpus-propus joint was positioned so that the bender muscle was stretched and the preparation was immersed in Bouin's fixative for 36 h. The bender muscle was removed from the carpus and stored in 70% ethanol.

Sarcomere length measurements were made from single muscle fibers that had been teased into myofibrils in a drop of 70% alcohol on a glass slide. The myofibrils were examined under Nomarski optics and the length of five successive sarcomeres was measured using a calibrated ocular micrometer. Measurements were made from five different myofibrils and an average sarcomere length value was calculated for the muscle fiber. Measurements were made for 100 fibers removed from each bender muscle.

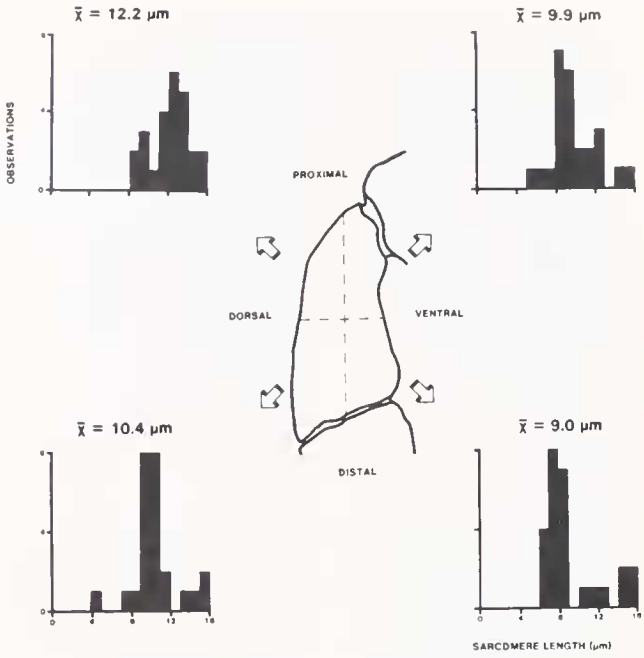

Figure 1. Sarcomere length measurements of 25 fibers removed from each quadrant of the bender muscle (inset diagram).

Results

The bender muscle is located in the carpopodite segment of the limb. In *P. crassipes*, the walking legs are compressed in the anterior-posterior direction, but the degree of compression of any one segment is not always constant. In the carpopodite, for example, the proximal region exhibits little compression and is essentially cylindrical (see Fig. 2). By contrast, the distal portion of the carpopodite is compressed to form flat anterior and posterior surfaces (see Figs. 3D and 4D). The inset of Figure 1 shows the orientation of the carpopodite. The limb is viewed from the anterior surface and is connected proximally to the meropodite and distally to the propodite. The other two surface are called dorsal and ventral.

Measurements made from 100 fibers removed from different areas in the bender muscle revealed an average sarcomere length of about 10.5 μm, with a range of 4 to 16 μm. In one preparation, the bender muscle was divided into four regions and measurements were made from each quadrant (Fig. 1—inset). The proximal-dorsal quadrant had fibers with the longest mean sarcomere

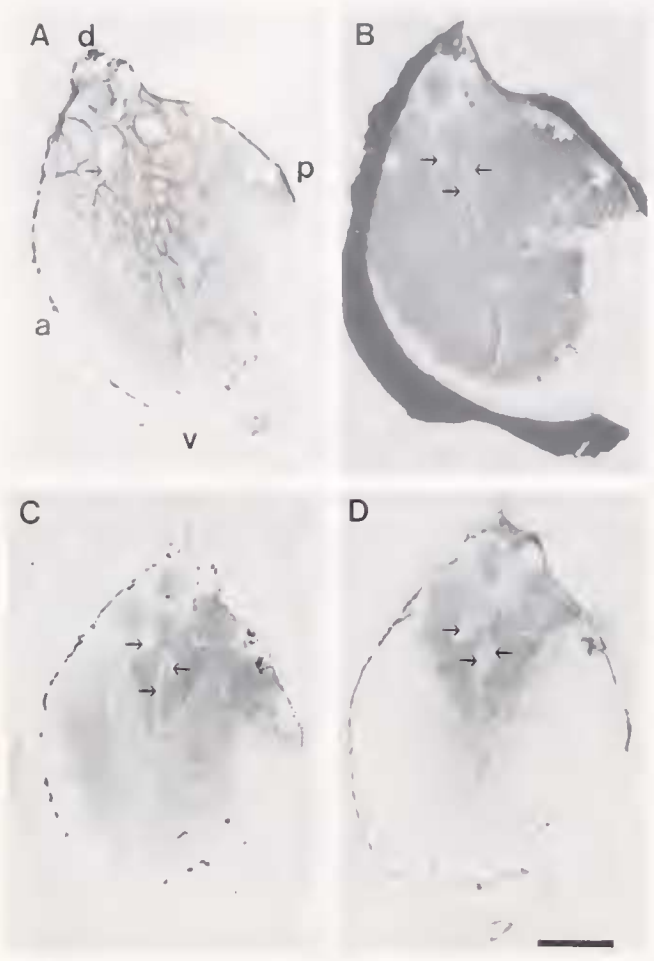

Figure 2. Histochemical properties of the bender muscle. Serial sections stained for NADH diaphorase activity (A), myofibrillar ATPase activity with alkaline (B) and acid (C) preincubation, and for polysaccharide content (D). a: anterior; d: dorsal; p: posterior; v: ventral. Calibration: 500 μm (A–C) and 415 μm (D).

length (12.2 μm; range 8 to 16 μm). The distal-dorsal quadrant contained a similar population of long-sarcomere fibers but also had some fibers with shorter (4 to 5 μm) sarcomeres. Fibers located on the ventral surface of the bender muscle had sarcomeres between 4 and 16 μm; the distal-ventral quadrant had a higher proportion of shorter-sarcomere (5 to 8 μm) fibers (Fig. 1).

Sections of the dissected carpopodite revealed that the bender muscle extends along the entire anterior surface and along some of the posterior surface of the segment (Fig. 2). The smaller stretcher muscle (which was removed prior to sectioning) is located along the posterior surface of the carpopodite and is confined to the ventral and central portion of the segment.

Bender muscle fibers can be differentiated by their contrasting histochemical properties. This can be seen in Figure 2, which shows four serial sections of the bender muscle taken at a level about one-third from the proximal end of the carpopodite. There is a population of muscle fibers that displays high NADH diaphorase activity, as seen by dark rings around the margin of individual fibers (Fig. 2A). In all preparations (n = 16), these types of fibers were located in the dorsal half of the muscle, on either side of the apodeme. With regard to myofibrillar ATPase activity, these same fibers stained poorly with alkaline preincubation (Fig. 2B) but well with acidic preincubation (Fig. 2C). Finally, these fibers had a high polysaccharide content (Fig. 2D).

Most of the other fibers in the bender muscle showed poor NADH diaphorase activity (Fig. 2A) and high alkaline myofibrillar ATPase activity (Fig. 2B). In all preparations (n = 16), staining for myofibrillar ATPase activity with acidic preincubation revealed that these fibers are not a homogeneous group. In Figure 2C there is one group of lighter staining fibers located towards the center of the muscle, and a second group of darker staining fibers around the peripheral margins of the muscle. A similar difference was also seen in sections stained for polysaccharide content (Fig. 2D). Fibers in the central portion of the muscle had a level of staining that was intermediate between fibers located more dorsally (dark staining) and fibers on the ventral margin of the muscle (light staining). Finally, in all preparations there were always some fibers that exhibited poor myofibrillar ATPase (at both pH levels) and NADH diaphorase activities (Fig. 2—arrows).

It is apparent that polysaccharide content may be correlated with the histochemical properties of most fibers in the bender muscle (Fig. 2). In all preparations (n = 16) fibers with high polysaccharide content (Fig. 2D) stained well for NADH diaphorase (Fig. 2A) and had high acid myofibrillar ATPase activity (Fig. 2C). Fibers with lower polysaccharide content (Fig. 2D) stained well for alkaline myofibrillar ATPase activity (Fig. 2B) and poorly for NADH diaphorase (Fig. 2A). We have used this correlation between fiber-type and polysaccharide content to differentiate fiber-types in the different regions of the bender muscle (Fig. 3); these observations were confirmed by examination of adjacent sections stained for NADH diaphorase activity (Fig. 4) and for myofibrillar ATPase activity with acid and alkaline pre-incubation (micrographs not shown).

Figures 3 and 4 each show four sections taken at different levels of the bender muscle; the inset numbers represent the level of the section of the carpopodite, with proximal as 0 and distal as 100. In the proximal portion of the muscle, fibers with high polysaccharide content and high NADH diaphorase activity form a "V-shaped wedge" around the apodeme in the dorsal portion of the muscle (Figs. 3A, B, and 4A, B). Intermediate staining fibers are located on either side of the "wedge," while poorly staining fibers are located mostly ventrally in the bender muscle (Fig. 3A, B).

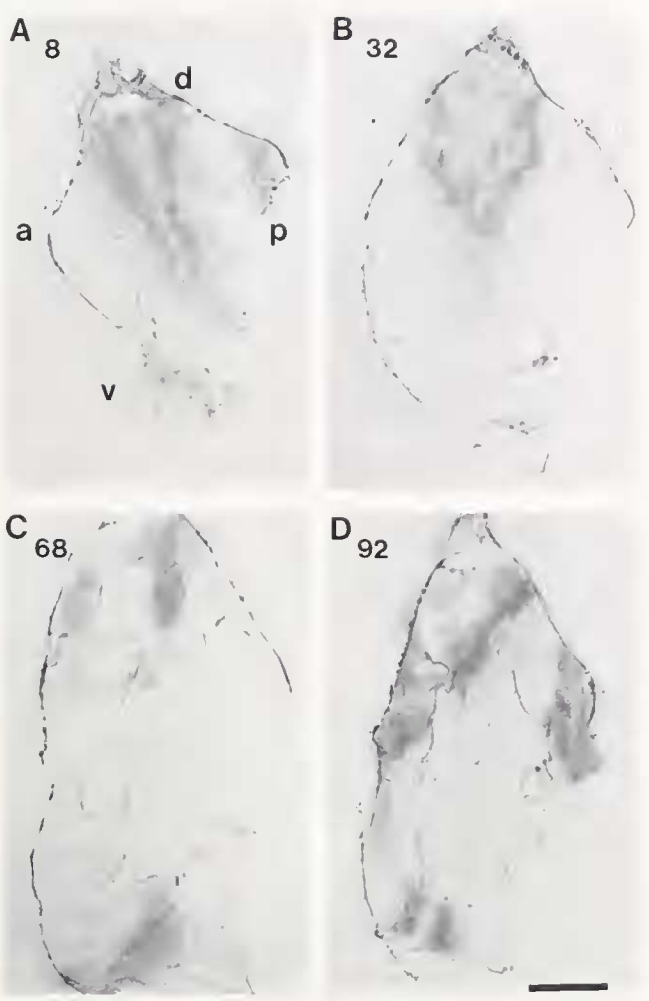

Figure 3. Polysaccharide content of bender muscle fibers. The inset numbers represent the level of sectioning in the muscle. 0 represents proximal, 100 represents distal. a: anterior; d: dorsal; p: posterior; v: ventral. Calibration: 500 μm.

In the distal half of the bender muscle, fibers with high polysaccharide content and high NADH diaphorase activity are located on the dorsal and ventral margins of the bender muscle (Figs. 3C, D, and 4C, D). Intermediate staining fibers are located mostly anteriorly, while poorly staining fibers are found mostly in the posterior-central portion of the bender muscle (Fig. 3C, D). The above staining profiles were consistent in all preparations (n = 16).

Discussion

The present study shows that the bender muscle in the walking limbs of *P. crassipes* is not composed of a uniform population of fibers. In other crustacean muscles a correlation has been found between fiber type, sarcomere length, NADH diaphorase activity and myofibrillar ATPase activity (Atwood, 1973; Ogonowski and Lang; 1979; Tse *et al.*, 1983; Maier *et al.*, 1984; Stephens *et al.*, 1985). Slow muscle fibers tend to have long sarcomeres (>10 μm), high NADH diaphorase activity, and high acid myofibrillar ATPase activity. In the proximal portion of the bender, fibers with long sarcomere lengths were found predominantly in the dorsal half of the muscle. The presumption that these fibers are slow is supported by the histochemical data, since there is a distinct population of muscle fibers with high NADH diaphorase activity and acid myofibrillar ATPase activity in this region (Fig. 2A, C). In the distal region of the muscle, however, presumptive slow fibers were concentrated in the dorsal, ventral and anterior portions of the muscle (Fig. 4C, D). It is interesting that NADH diaphorase activity in individual slow muscle fibers is confined to the peripheral margin (Figs. 2A and 4). A similar observation has been made in the tails of lobster (Ogonowski and Lang, 1979) and crab (Stephens *et al.*, 1985) and indicates that

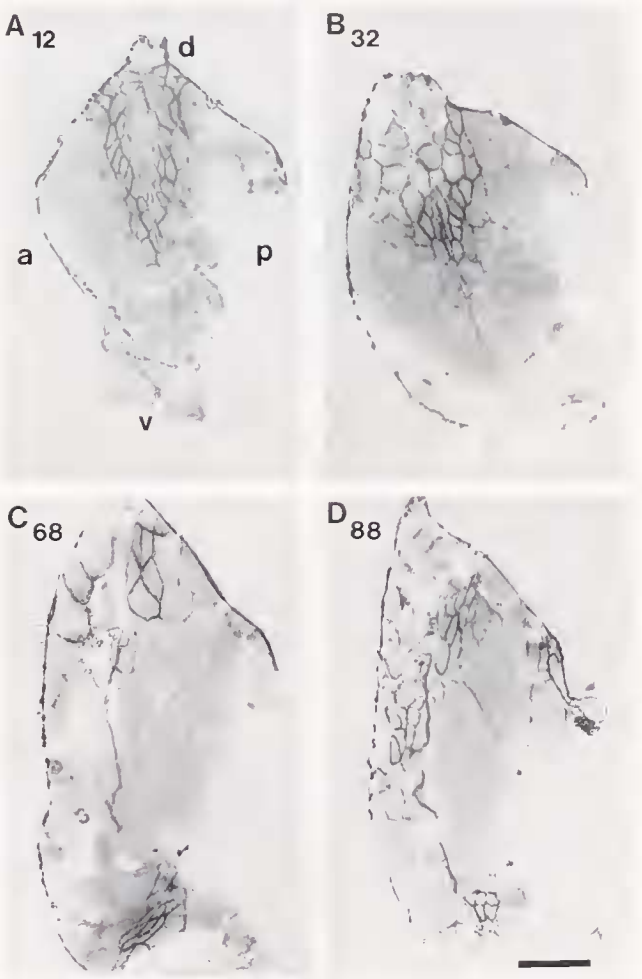

Figure 4. NADH diaphorase activity of bender muscle fibers. The inset numbers represent the level of sectioning in the muscle. 0 represents proximal, 100 represents distal. a: anterior; d: dorsal; p: posterior; v: ventral. Calibration: 500 μm.

the mitochondria containing this enzyme are concentrated in the peripheral margins of the slow fibers.

In many crustacean muscles, fast fibers have short sarcomeres (<4 μm), low NADH diaphorase activity and high alkaline myofibrillar ATPase activity. Our observations revealed no sarcomeres less than 4 μm in length (Fig. 1), indicating that the bender muscle contains no fast fibers. The remaining fibers in the bender muscle are therefore assumed to be intermediate-type fibers. The intermediate-type fibers appear to be concentrated ventrally in the proximal portion of the muscle (Fig. 2), and posteriorly in the distal portion of the muscle (Figs. 3 and 4).

In the present study we have found that the polysaccharide content is highest in slow fibers (Fig. 2). A similar correlation has been made between polysaccharide content and fiber-type in crab swimming muscles (Tse *et al.*, 1983). Furthermore, based on polysaccharide content there appear to be two types of intermediate fibers. Moreover, the distribution of the fibers with different polysaccharide content appears to be in line with those that exhibit contrasting acid ATPase activity (Figs. 2C, D). Fibers adjacent to the slow fibers have higher polysaccharide content and have a lighter staining profile for ATPase activity. Paradoxically, in some sections there were one or more fibers with intermediate polysaccharide content, and low NADH diaphorase and myofibrillar ATPase activities (Fig. 2—arrows). We have not been able to typify these fibers.

It may be argued that the polysaccharide content of the different fibers was influenced by the dissection procedure prior to freezing of the tissue. The dissection may have selectively stimulated one axon so that the innervated fibers could have been activated and thus could have decreased or depleted their polysaccharide stores. To examine this possibility, we froze several limbs immediately after autotomy. Although the presence of cuticle decreased the quality of the sections, no differences between the staining profiles of these sections and those from dissected preparations were observed (McDermott, unpub. observations). We conclude that the difference in the polysaccharide content of the various muscle fibers is not artifact.

Acknowledgments

This work was funded by a grant from the Research Corporation, the technical assistance of Ms. Louise diCola is gratefully acknowledged.

Literature Cited

Atwood, H. L., 1973. An attempt to account for the diversity of crustacean muscle. *Int. Zool.* 13: 357–378.

Atwood, H. L., and J. M. Wojtowicz, 1986. Short-term and long-term plasticity and physiological differentiation of crustacean motor synapses. *Int. Rev. Neurobiol.* **28:** 275–361.

Barany, M., and R. I. Close, 1971. The transformation of myosin in cross-innervated rat muscles. *J. Physiol.* **213:** 455–474.

Buller, A. J., and D. M. Lewis, 1965. Further observations on mammalian cross-innervated skeletal muscle. *J. Physiol.* **178:** 343–358.

Buller, A. J., J. C. Eccles, and R. M. Eccles, 1960. Interaction between motor neurons and muscles in respect to the characteristic speed of their response. *J. Physiol.* **150:** 417–439.

Buller, A. J., W. F. H. M. Mommaerts, and K. Seraydarian, 1969. Enzymic properties of myosin in fast and slow twitch muscle of the cat following cross-reinnervation. *J. Physiol.* **205:** 581–597.

Guth, L., 1968. Trophic influences of nerve on muscle. *Physiol. Rev.* **48:** 645–687.

Gutmann, P. V., 1976. Neurotrophic relations. *Ann. Rev. Physiol.* **38:** 177–216.

Lang, F., M. M. Ogonowski, W. J. Costello, R. Hill, B. Roehrig, K. Kent, and J. J. Sellars, 1980. Neurotrophic influence on lobster skeletal muscle. *Science* **207:** 325–327.

Lnenicka, G. A., and H. L. Atwood, 1985. Age-dependent long-term adaptation of crayfish phasic motor axon synapses to altered activity. *J. Neurosci.* **5:** 459–467.

Lnenicka, G. A., H. L. Atwood, and L. Marin, 1986. Morphological transformation of synaptic terminals of a phasic motoneuron by long-term tonic stimulation. *J. Neurosci.* **6:** 2252–2258.

Lillie, R. D., and H. M. Fullman, 1976. *Histopathologic Technic and Practical Histochemistry* 4th Ed. McGraw-Hill, New York.

Lomo, T., R. H. Westgaard, and H. A. Dahl, 1974. Contractile properties of muscle: control by pattern of muscle activity in the rat. *Proc. R. Soc. Lond. (Biol.)* **187:** 99–103.

Luff, A. R., 1975. Dynamic properties of fast and slow skeletal muscles in the cat and rat following cross-innervation. *J. Physiol.* **248:** 83–96.

Maier, L., W. Rathmayer, and D. Pette, 1984. pH lability of myosin ATPase activity permits discrimination of different muscle fibre types in crustaceans. *Histochemistry* **81:** 75–77.

O'Connor, K., P. J. Stephens, and J. M. Leferovich, 1982. Regional distribution of muscle fiber types in the asymmetric claws of Californian snapping shrimp. *Biol. Bull.* **163:** 329–336.

Ogonowski, M. M., and F. Lang, 1979. Histochemical evidence for enzyme differences in crustacean fast and slow muscle. *J. Exp. Zool.* **207:** 143–151.

Padykula, H. A., and A. Hermann, 1955. Factors affecting the activity of adenosine triphosphate and other phosphotases as measured by histochemical techniques. *J. Histochem. Cytochem.* **3:** 161–169.

Pahapill, P. A., G. A. Lnenicka, and H. L. Atwood, 1986. Neuronal experience modifies synaptic long-term facilitation. *Can. J. Physiol. Pharmacol.* **64:** 1052–1054.

Pette, D., 1984. Activity-induced fast to slow transitions in mammalian muscle. *Med. Sci. Sports. Exercise* **16:** 517–528.

Sreter, F. A., A. R. Luff, and J. Gergely, 1975. Effect of cross-reinnervation on physiological parameters and on properties of myosin and sarcoplasmic reticulum of fast and slow muscles of the rabbit. *J. Gen. Physiol.* **66:** 811–821.

Stephens, P. J., J. M. Leferovich, and P. Klainer, 1985. Neuromuscular relationships in the abdomen of the Californian shore crab *Pachygrapsus crassipes*. *J. Neurobiol.* **16:** 127–136.

Tse, F. W., C. K. Govind, and H. L. Atwood, 1983. Diverse fiber composition of swimming muscles in the blue crab, *Callinectes sapidus*. *J. Can. Zool.* **61:** 52–59.

Wiersma, C. A. G., and S. H. Ripley, 1952. Innervation patterns of crustacean limbs. *Comp. Oecol.* **2:** 391–405.